# ANALYSIS OF LIPOPHILIC PIGMENTS FROM A PHOTOTROPHIC MICROBIAL MAT COMMUNITY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.

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Abstract

An assay for lipophilic pigments in phototrophic microbial mat communities using reverse phase-high performance liquid chromatography was developed which allows the separation of 15 carotenoids and chloropigments in a single 30 min program. Lipophilic pigments in a laminated mat from a commercial salina near Laguna Guerrero Negro, Baja California Sur, Mexico reflected their source organisms. Myxoxanthophyll, echinenone, canthaxanthin, and zeaxanthin were derived from cyanobacteria; chlorophyll c, and fucoxanthin from diatoms; chlorophyll a from cyanobacteria and diatoms; bacteriochlorophylls a and c, bacteriophaeophytin a, and $\gamma$-carotene from photosynthetic bacteria; and $\beta$-carotene from a variety of phototrophs. Sensitivity of detection was 0.6 - 6.1 ng for carotenoids and 1.7 - 12 ng for most chloropigments. This assay represents a significant improvement over previous analyses of lipophilic pigments in microbial mats and promises to have a wider application to other types of phototrophic communities.

Key Words: carotenoids, chlorophylls, cyanobacteria, Chloroflexus, microbial mats.

Introduction

Microbial mats are often comprised of consortia of procaryotic and eucaryotic phototrophic microorganisms [1] and thus contain complex mixtures of lipophilic pigments [2,3]. Laminated microbial mat communities have been described in a salina near Laguna Guerrero Negro, Baja California Sur, Mexico [4,5]. These mats are dominated by Microcoleus chthonoplastes, a cosmopolitan mat-building cyanobacterium, described, for example, in microbial mats from Solar Lake, Israel [6,7]. At Guerrero Negro, M. chthonoplastes is found together with Oscillatoria sp., unicellular cyanobacteria, green gliding bacteria (Chloroflexaceae), diatoms, as well as non-phototrophic microorganisms including Beggiatoa and sulfate-reducing bacteria.

As part of an effort to identify light absorbing pigments and to search for potential lipid biomarkers in microbial mats, we developed an assay using reverse phase-high performance liquid chromatography (HPLC) to separate, identify, and quantify principal lipophilic pigments. Although a number of assays utilizing HPLC have been reported for separating pigments from marine phytoplankton including diatoms and dinoflagellates [8,9], the separation of the wide variety of pigments found in photosynthetic microorganisms in microbial mats posed a special problem. Using a modification of an assay for cyanobacterial pigments [10], we were able to separate chlorophylls and bacteriochlorophylls, their corresponding phaeophytins, and carotenoids from this natural community of mixed phototrophs in a single 30 min program. We believe that this is a significant

improvement over previously reported assays of lipophilic pigments in microbial mats [3].

Methods

Microbial mats were collected from the bottom of a concentrating pond (Pond 5, 87 o/oo) in a commercial salina (Exportadora de Sal) at Guerrero Negro in June, 1986. Cores were taken with cut-off 30 ml plastic syringes, the top 9 mm was sectioned into approximately 0.5 or 1.0 mm sections, and stored frozen in the dark until their return to NASA-Ames Research Center. Samples were lyophilized, homogenized and extracted overnight in 90% acetone at 0°C in the dark, then re-extracted in fresh acetone for 1-2 hours. Further extraction with 90% acetone or with chloroform/methanol (2:1 v/v) failed to produce any additional color. Extracts were filtered through a GF/C to remove cell debris, then filtered again through a 0.5 um pore size Millipore FH filter. Extracts were kept in the dark at all times, and chromatographed within several hours of filtration. Concentration of extracts with diethyl ether or Sep-Pak columns [11] was unnecessary, minimizing sample handling. Separations were performed on a Hewlett Packard 1084B high performance liquid chromatograph on a 250 mm X 4.6 mm ODS (C-18) Hypersil column with 5 um packing ( Alltech Associates). A Whatman CSK guard column filled with Whatman ODS (Co:Pel) 30-38 um packing was used to protect the analytical column. The ultraviolet-visible wavelength absorbance detector which has a built-in scanning mode (250-550 nm) was set at 440 nm (550 nm reference) for our analyses.

Initially, we tried to separate microbial mat pigments using

a gradient program reported by Paerl et al [10]. Briefly, the mobile phase of solvent A (90:10 v/v methanol:acetonitrile) and solvent B (100% acetone) was regulated at flow rate of 1.5 ml·min$^{-1}$ according to the following program: 0-7 min 100% A, 7-11 min linear increase to 60% B, 11-20 min 60% B, 20-28 min linear decrease to 100% A. Finding this program to be unsatisfactory for our microbial mat samples, we modified the existing program in the following manner. First , de-ionized water was added to Solvent A for a final concentration of 5% (v/v) to increase polarity; acetonitrile was adjusted to 13% (v/v) and methanol was 83% (v/v). Solvent B remained as 100% acetone. Second, an ion-pairing agent, tetrabutyl ammonium acetate (TBAA; Alfa Products) was added to improve the separation of chloropigments [8]. However, it was necessary to decrease the amount of TBAA previously recommended [8] because the TBAA caused precipitation which reduced chromatographic resolution. We found that 3.85 g TBAA with 7.5 g ammonium acetate in 100 mls deionized water, prior to its addition to Solvent A, was sufficient to separate chloropigments without reducing peak resolution. Third, to improve the sensitivity of detection of bacteriochlorophyll *a* and bacteriophaeophytin *a* which absorb maximally at 360 nm, and phaeophytin *a* which absorbs maximally at 410 nm, we programmed the detector for changes in wavelength corresponding to the retention times for these peaks . The solvent program is listed in Table 1. Organic solvents (Burdick and Jackson, HPLC grade) were bubbled with $N_2$ or He prior to use; the flow rate remained at 1.5 ml·min$^{-1}$. The column was rinsed daily with deionized, degassed

water followed by 100 % methanol to avoid the build-up of TBAA salts on the column; the column was stored in methanol.

Pigments from the microbial mat were identified by characteristic absorption maxima in organic solvents using ultraviolet-visible spectroscopy and by co-chromatography with standards [8,12]. $\beta$-carotene, chlorophyll *a*, and bacteriochlorophyll *a* were obtained from Sigma Chemical Co. Phaeophytins were produced from their corresponding chlorophylls by acidification. Zeaxanthin, fucoxanthin, canthaxanthin and echinenone were donated by Hoffmann-LaRoche Co.; these carotenoids were purified immediately prior to use by thin layer chromatography (TLC) [12,13]. Using TLC, we also isolated myxoxanthophyll from cyanobacteria, chlorophyll *c* from the diatom *Navicula saprophyta*, and $\gamma$-carotene and bacteriochlorophyll *c* from a culture of *Chloroflexus aurantiacus*. Pigments were quantified by comparison with standard curves of chloropigments (Figure 1) and carotenoids (Figure 2). All standard curves were linear with correlation coefficients of >0.95 ($P<0.05$) [14].

## Results and Discussion

From a review of the scientific literature, we found that the Paerl et al [10] program provided the best overall separation of cyanobacterial pigments. Other programs, such as Mantoura and LLewellyn [8], failed to clearly resolve echinenone, a common cyanobacterial carotenoid, and chlorophyll *a*. The Paerl et al program, however, failed to separate completely the more polar pigments such as fucoxanthin, zeaxanthin, myxoxanthophyll, and chlorophyll *c* in the mixed phototrophic populations from the

microbial mats (Figure 3A). By modifying this program as described in our methods, we were able to separate the more polar xanthophylls and chlorophyll *c* (peaks 1-6) and to increase the sensitivity of detection of bacteriochlorophyll *a*, bacteriophaeophytin *a*, and phaeophytin *a* (Fig. 3B - peaks 8, 12, and 15, respectively). Retention times, absorption maxima, published extinction coefficients, and sensitivity of detection (Signal/Noise=2) are shown in Table 2. Replication between runs was within 3%. Carotenoids could be detected to 0.6 - 6.1 ng. Chloropigments, with the exception of bacteriochlorophyll *c*, were detected to 1.7 - 12 ng. Bacteriochlorophyll *c*, however, chromatographed into four separate peaks; these separate fractions may reflect the different functional types of bacteriochlorophyll *c* that have been found in the chlorosomes of *Chloroflexus* [20].

The lipophilic pigments isolated from the Guerrero Negro Pond 5 microbial mat reflected their source microorganisms (Table 3). Our pigment analyses were complemented by parallel studies of the microbial mats using light and transmission electron microscopy [21]. As certain carotenoids are specific to a single taxon, carotenoids can be especially useful in chemotaxonomy [22]. Myxoxanthophyll, a carotenoid specific to cyanobacteria, was probably contributed by *M. chthonoplastes*, *Oscillatoria* sp. and unicellular cyanobacteria; echinenone, zeaxanthin and canthaxanthin are also commonly found in cyanobacteria. Fucoxanthin and chlorophyll *c* originated from diatoms in the microbial mats. Bacteriochlorophylls *a* and *c*, and $\gamma$-carotene are part of the photosynthetic apparatus of *Chloroflexus* [20,23]. Bacteriochlorophyll *a* may also have been derived from purple

sulfur bacteria which were present but in very low abundance [16]. $\beta$-carotene, whose primary function appears to be photoprotection, can be found in most phototrophic mat organisms.

Lipophilic pigments in microbial mats apparently play roles in both light absorption and photoprotection. In vivo attenuation spectra on microbial mats from Laguna Guerrero Negro, Pond 5 by Jorgensen et al [5] showed absorption by chlorophyll a (430 nm, 670 nm), carotenoids (440-550 nm) as well as by water-soluble phycobiliproteins (620 nm). Based on action spectra for photosynthesis, chlorophyll a and carotenoids appeared to be important for light-harvesting in the surface film of diatoms. By contrast, photosynthesis by M. chthonoplastes was dependent upon light-harvesting by phycobiliproteins with carotenoids serving primarily a photoprotective function.

HPLC is a significant improvement over spectrophotometric analysis, where overlapping absorbance spectra result in poor precision, and TLC, which is time-consuming and relatively insensitive [8]. Stal et al [3] used phase separation and TLC to isolate and purify chlorophylls and bacteriochlorophylls from a laminated microbial mat community; however, their technique was limited in resolution and accuracy due to an incomplete separation of pigments. Korthals and Steenburgen [24] applied HPLC to the analysis of phytoplankton cells and phototrophic sulfur bacteria in Lake Vechten. Pigments including myxoxanthophyll and bacteriophaeophytin a, however, were not isolated in their samples, nor were they able to resolve $\gamma$- and $\beta$-carotene. We believe our HPLC program will be useful in the determination of

lipophilic pigments from a wide range of phototrophic microbial communities. For example, this program has recently been successfully applied to separate pigments including chlorophyll b, phaeophytin b and alloxanthin in microbial populations dominated by green algae, cryptomonads, chrysophytes and diatoms [25].

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Table 1. Solvent program for high performance liquid chromatography (HPLC) of lipophilic pigments. See text for description of solvents. Reference wavelength is 550 nm.

| Time (min) | % Solvent B | Wavelength (nm) |
|---|---|---|
| 0.00 | 5 | 440 |
| 10.00 | 28 | |
| 11.00 | | 360 |
| 12.50 | | 440 |
| 20.00 | 28 | |
| 22.00 | 50 | |
| 23.50 | | 360 |
| 25.00 | 60 | |
| 25.20 | | 440 |
| 29.00 | 70 | 410 |
| 30.00 | 5 | |
| 31.00 | | 440 |
| 35.00 | 5 | |
| STOP | | |

Table 2. Retention times in HPLC program for lipophilic pigments found in microbial mat communities from Laguna Guerrero Negro.

| Peak No. | Retention Time (min) | Pigment | Absorbance Maxima (nm) | Sensitivity (ng) | $E^{1\%}$ [a] | Solvent | Reference |
|---|---|---|---|---|---|---|---|
| 1 | 2.34 | chlorophyll *c* | 445 | 1.7 | 3460 | methanol | 8 |
| 2 | 3.16 | fucoxanthin | 446,470 | 4.9 | 1016 | ethanol | 8 |
| 3 | 4.29 | myxoxanthophyll | 470 | 0.6 | 1253 | ethanol | 8 |
| 4 | 5.70 | zeaxanthin | 452,474 | 1.6 | 2000 | methanol | 8 |
| 5 | 6.28 | unknown | | --- | ---- | ----- | -- |
| 6 | 8.00 | canthaxanthin | 478 | 3.7 | 2000 | ethanol | 8 |
| 7 | 10.01 | bacterio-chlorophyll *c* | (400),(424), 440 | 43.6 | 860 | methanol | 15 |
| 8 | 12.30 | bacterio-chlorophyll *a* | 368 | 8.7 | 923 | acetone | 16 |
| 9 | 15.28 | chlorophyll *a*' | 415,430 | --- | 687 | 90% acetone | 17 |
| 10 | 18.15 | echinenone | 465 | 6.1 | 1881 | ethanol | 8 |
| 11 | 18.90 | chlorophyll *a* | 415,430 | 6.5 | 687 | 90% acetone | 17 |
| 12 | 22.98 | bacterio-phaeophytin *a* | 368 | 11.8 | 509 | acetone: methanol (7:2) | 18 |
| 13 | 27.03 | $\gamma$-carotene | (440), 476, 492 | 1.1 | 2760 | hexane | 19 |
| 14 | 27.72 | $\beta$-carotene | 453,476 | 3.2 | 2209 | ethanol | 8 |
| 15 | 28.03 | phaeophytin *a* | (375),410 | 2.4 | 713 | ether | 17 |

[a] specific extinction coefficients at 440 nm except for bacteriochlorophyll *c* (668 nm), bacteriochlorophyll *a* (772 nm), and bacteriophaeophytin *a* (747 nm).

Table 3. Lipophilic pigments from phototrophic microorganisms in a layer of laminated microbial mat from a 1-2 mm depth.

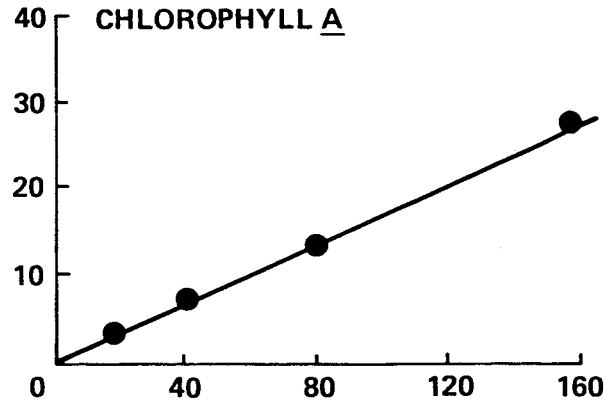
| Pigment | ng · mg dry weight$^{-1}$ | Possible Source |
|---|---|---|
| chlorophyll *c* | 1.2 | diatoms |
| fucoxanthin | 42.5 | diatoms |
| myxoxanthophyll | 41.3 | cyanobacteria |
| zeaxanthin | 23.5 | cyanobacteria |
| canthaxanthin | 9.5 | cyanobacteria |
| bacteriochlorophyll *c* | trace | *Chloroflexus* |
| bacteriochlorophyll *a* | 59.2 | photosynthetic bacteria |
| chlorophyll *a*' | 40.7 | cyanobacteria, diatoms |
| echinenone | 7.5 | cyanobacteria |
| chlorophyll *a* | 368.3 | cyanobacteria, diatoms |
| bacteriophaeophytin *a* | trace | photosynthetic bacteria |
| $\gamma$-carotene | 13.1 | *Chloroflexus* |
| $\beta$-carotene | 138.0 | cyanobacteria, diatoms, photosynthetic bacteria |
| phaeophytin *a* | not detectable | cyanobacteria, diatoms |

Figure Legends

Figure 1. Standard curves for six chloropigments from phototrophic mat microorganisms including cyanobacteria (chlorophyll _a_, phaeophytin _a_), diatoms (chlorophylls _a_ and _c_, phaeophytin _a_), and photosynthetic bacteria (bacteriochlorophylls _a_ and _c_, bacteriophaeophytin _a_). Absorbance of chlorophylls _a_ and _c_, and bacteriochlorophyll _c_ was measured at 440 nm, phaeophytin _a_ at 410 nm, and bacteriochlorophyll _a_ and bacteriophaeophytin _a_ at 360 nm. Curves were fitted with a linear regression, $r>0.95$.

Figure 2. Standard curves for eight carotenoids from phototrophic mat microorganisms including cyanobacteria (myxoxanthophyll, zeaxanthin, canthaxanthin, echinenone, $\beta$-carotene). diatoms (fucoxanthin, $\beta$-carotene), and Chloroflexus ($\gamma$- and $\beta$-carotene) at 440 nm. Curves were fitted with a linear regression, $r>0.95$.

Figure 3. Absorbance chromatograms of lipophilic pigments from a laminated microbial mat in Laguna Guerrero Negro. Pigments from cyanobacteria, diatoms, and photosynthetic bacteria were present in this layer at a 1-2 mm depth in the mat. Peaks are identified in Table 2. (A) Paerl et al (1983) program. 50 uL was injected at 0.12 absorbance units full scale. (B) Modified program described in this study. 50 uL was injected at 0.06 absorbance units full scale.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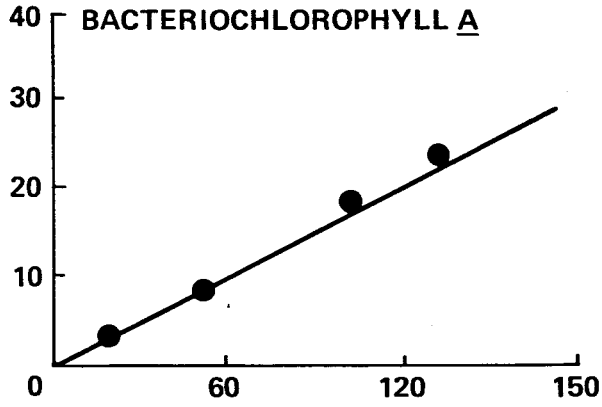
30
20
10
0
40
80
120
160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40
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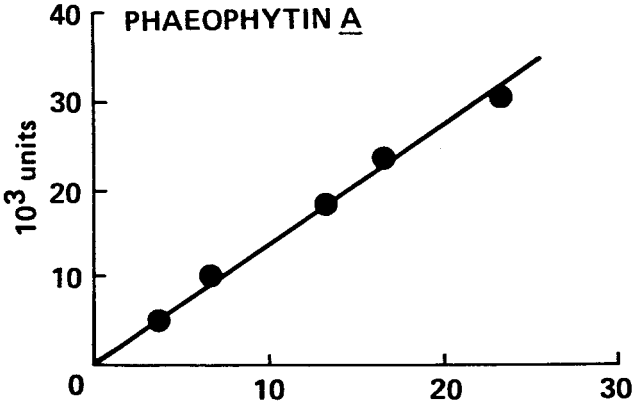
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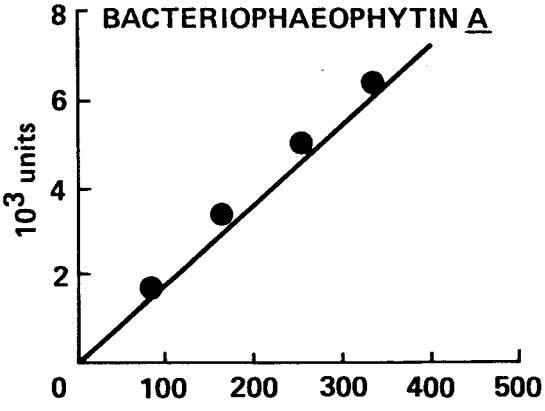
BACTERIOPHAEOPHYTIN A
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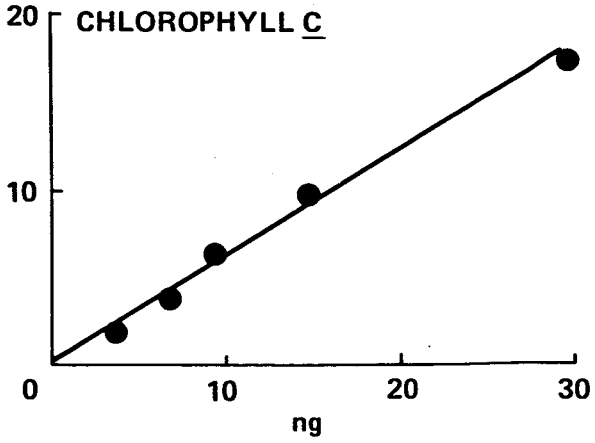
400
500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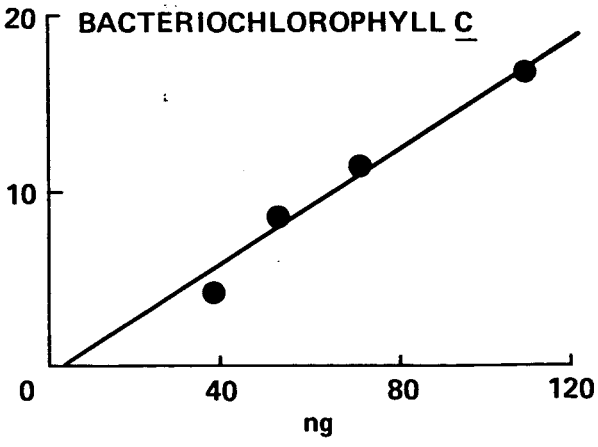
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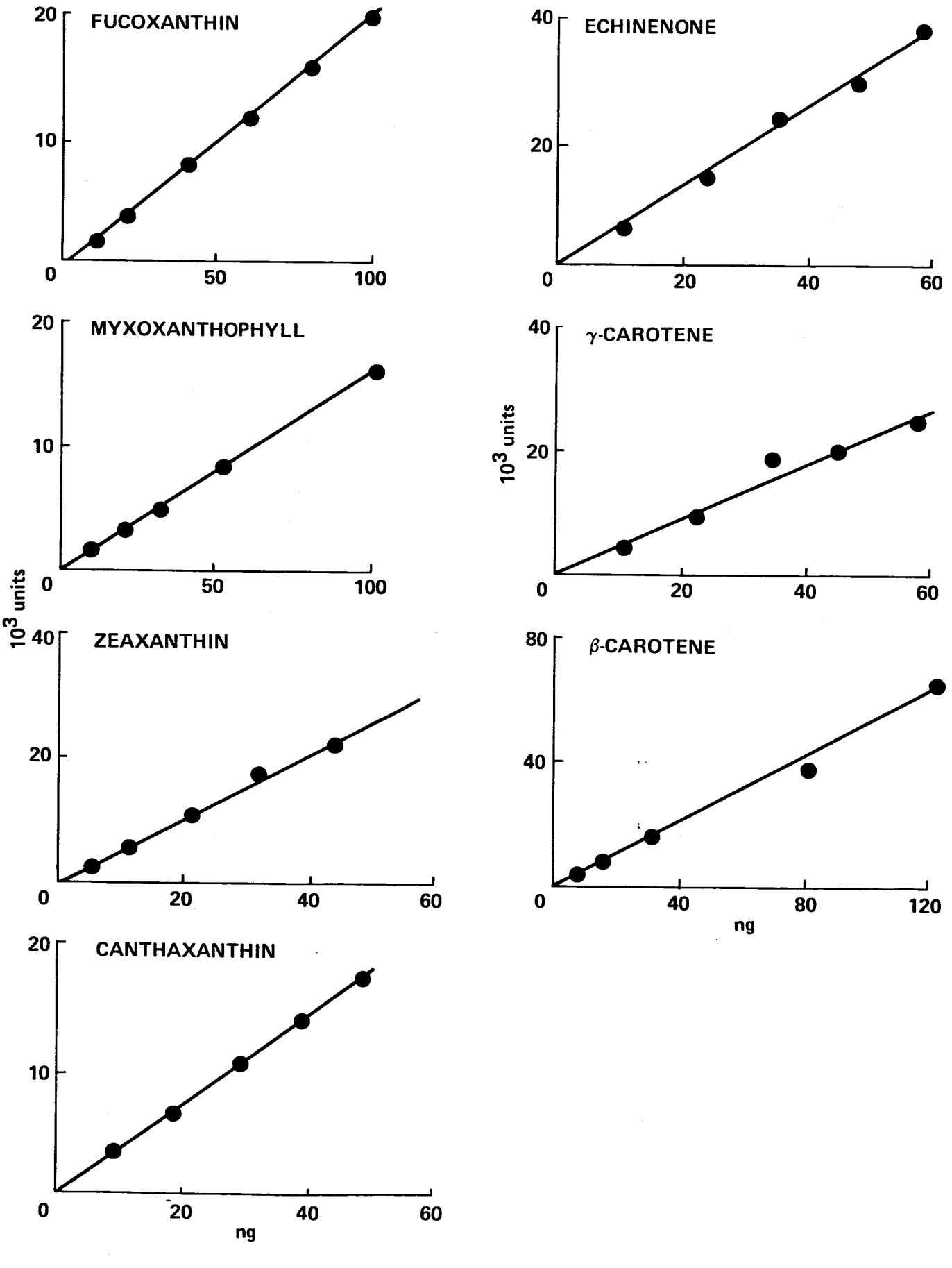
FUCOXANTHIN
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60
MYXOXANTHOPHYLL
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0
50
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γ-CAROTENE
40
20
0
20
40
60
$10^3$ units
ZEAXANTHIN
40
20
0
20
40
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β-CAROTENE
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CANTHAXANTHIN
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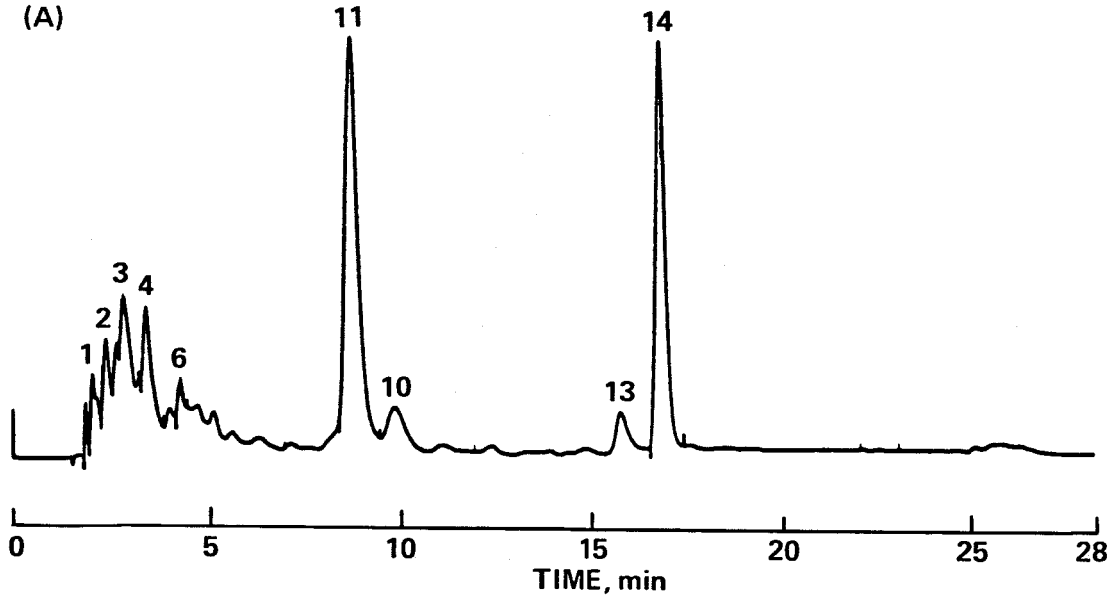
(A)
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14
3
4
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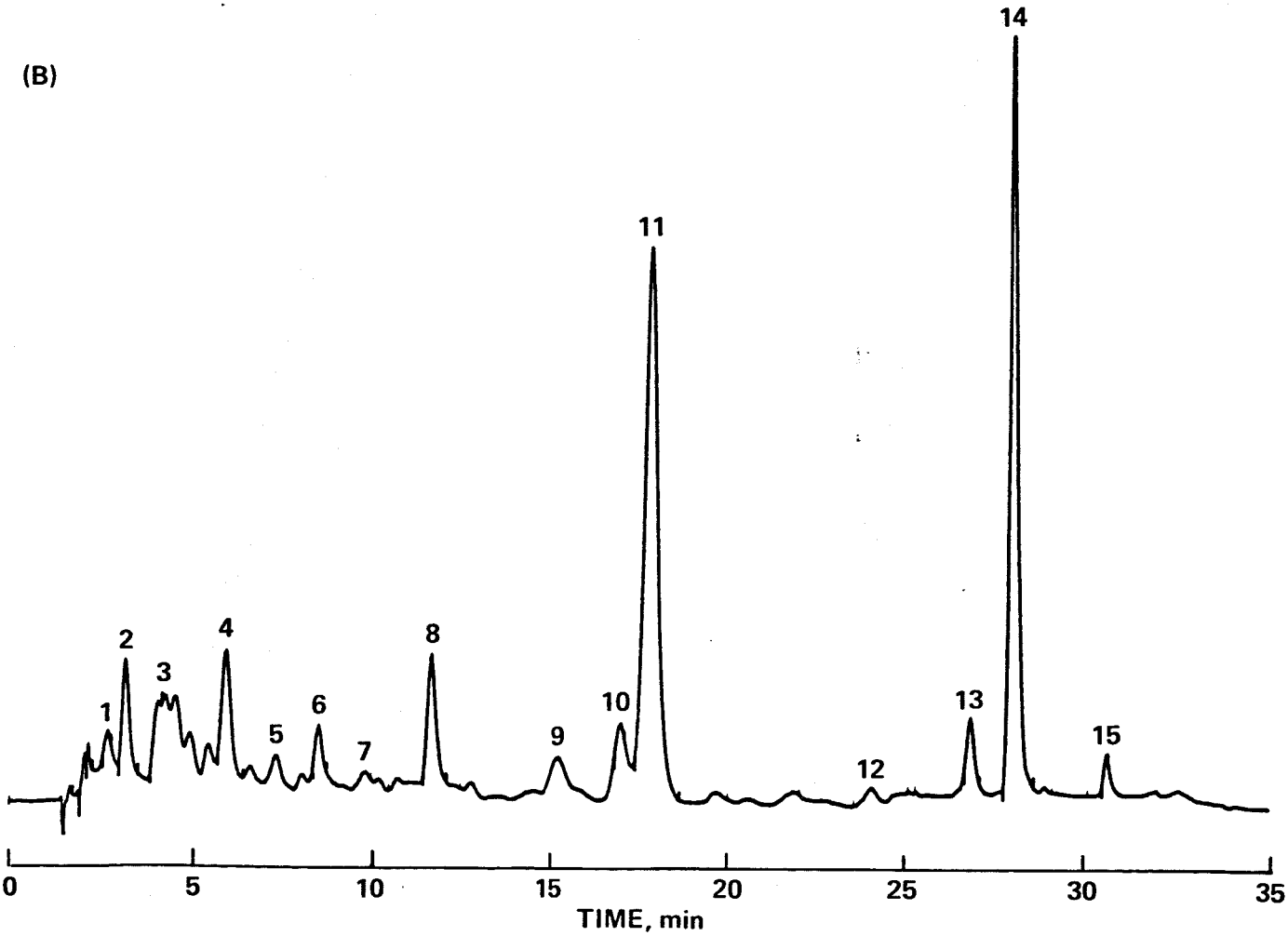
25
28
TIME, min


(B)
14
11
2
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8
3
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9
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25
30
35
TIME, min